

# Normal Platelet Activation Profile in Patients with Peripheral Arterial Disease on Aspirin

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## Regular Article

Normal Platelet Activation Profile in Patients with Peripheral Arterial Disease on Aspirin<sup>☆</sup>

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## ABSTRACT

**Background:** Peripheral arterial disease (PAD) is a progressive vascular disease associated with a high risk of cardiovascular morbidity and death. Antithrombotic prevention is usually applied by prescribing the antiplatelet agent aspirin. However, in patients with PAD aspirin fails to provide protection against myocardial infarction and death, only reducing the risk of ischemic stroke. Platelets may play a role in disease development, but this has not been tested by proper mechanistic studies. In the present study, we performed a systematic evaluation of platelet reactivity in whole blood from patients with PAD using two high-throughput assays, *i.e.* multi-agonist testing of platelet activation by flow cytometry and multi-parameter testing of thrombus formation on spotted microarrays.

**Methods:** Blood was obtained from 40 patients (38 on aspirin) with PAD in majority class IIa/IIb and from 40 age-matched control subjects. Whole-blood flow cytometry and multiparameter thrombus formation under high-shear flow conditions were determined using recently developed and validated assays.

**Results:** Flow cytometry of whole blood samples from aspirin-treated patients demonstrated unchanged high platelet responsiveness towards ADP, slightly elevated responsiveness after glycoprotein VI stimulation, and decreased responsiveness after PAR1 thrombin receptor stimulation, compared to the control subjects. Most parameters of thrombus formation under flow were similarly high for the patient and control groups. However, *in vitro* aspirin treatment caused a marked reduction in thrombus formation, especially on collagen surfaces. When compared per subject, markers of ADP- and collagen-induced integrin activation (flow cytometry) strongly correlated with parameters of collagen-dependent thrombus formation under flow, indicative of a common, subject-dependent regulation of both processes.

**Conclusion:** Despite of the use of aspirin, most platelet activation properties were in the normal range in whole-blood from class II PAD patients. These data underline the need for more effective antithrombotic pharmacoprotection in PAD.

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## Introduction

Peripheral arterial disease (PAD) is a systemic vascular disorder with manifestations of atherosclerosis in particular in the lower extremities, resulting in obstruction of the arterial blood flow, and strongly associated with cardiovascular events [1–3]. In the Western world, a

considerable part of the elderly population appears to have characteristics of PAD, albeit the affected subjects often remain asymptomatic [4,5]. Symptomatic patients, already at an early stage (Fontaine class IIa/IIb), suffer from intermittent claudication and cannot walk a long distance without feeling pain [2]. At later stages (Fontaine classes III/IV), PAD patients progressively suffer from chronic and critical leg ischemia [2].

While it is recognized that systemic atherosclerosis is one of the underlying diseases of PAD, the pathophysiology of this disease is still not well understood. One of the early concepts was that aberrant coagulation, inflammation and platelet activity in these patients lead to ongoing thrombogenesis in the lower extremities [6]. In agreement with this, markers of systemic coagulation such as levels of D-dimer are increased in patients with PAD, but it is unclear whether this also reflects causality

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[7–10]. Consistently, also plasma levels of the inflammation marker C-reactive protein are elevated [10,11]. There is also ample evidence in the literature for increased platelet activation and aggregation in PAD [12–15], although this is not an undisputed finding [8,16,17]. The role of platelets in PAD may indeed be complex. On the one hand, antiplatelet treatment with aspirin, inhibiting thromboxane formation, is a standard therapy for PAD patients, with inhibitors of the platelet P2Y<sub>12</sub> receptors (clopidogrel, prasugrel) as effective alternatives [18]. On the other hand, there is evidence that in these patients, even after aspirin treatment, platelet functions remain high [19], platelet-monocyte aggregates are still formed [20], and platelet cleavage products such as soluble CD40L accumulate in the plasma [21]. Since PAD in many patients is a progressive disease with high cardiovascular risk, the question is whether residual high on-treatment platelet activity may contribute to the disease progression.

In recent years, a number of whole-blood based tests for detailed and overall platelet phenotyping in bleeding and thrombosis have been developed and validated. Plate-wells based flow cytometric tests allow simultaneous determination of key platelet responses, i.e. fibrinogen binding (integrin  $\alpha_{IIb}\beta_3$  activation) and secretion (P-selectin exposure) in response to a panel of receptor agonists [22,23]. Such flow cytometric tests are advantageous in detecting changed platelet activation in thrombotic patients [24]. Furthermore, flow chamber devices for whole-blood perfusion over a thrombogenic surface like collagen have appeared to be highly valuable in detecting a loss or gain of platelet function, supporting their use as *ex vivo* assays reflecting arterial thrombus formation [25–27]. Recently, we have extended this method into a multi-parameter platelet function test to assess thrombus formation on arrays of microspotted platelet-adhesive surfaces [28].

In the present paper, we used both high-throughput whole-blood assays, i.e. multi-agonist testing of platelet activation by flow cytometry and multi-parameter testing of thrombus formation on microarray spots, for detailed evaluation of the platelets in patients with PAD receiving aspirin. The data show that platelet function in these patients is in general high despite aspirin treatment, thus not pointing to a decreased prothrombotic propensity.

## Methods

### Patients and Controls

Included were 40 patients with established PAD. Patients were selected based on a decrease in the ankle brachial index  $<0.9$  [29,30] and most of them suffered from a stage IIa/IIb disorder according to the Fontaine classification [2]. As a control group, 40 healthy control subjects were included with similar age and gender. Fitness (i.e., non-disease state) of the control subjects was assessed with the Edinburgh claudication questionnaire [31]. Medication use was checked in all patient and control subjects by personal interview. Exclusion criteria for patients and controls were: proved coagulation or chronic inflammatory disorders, active infection, malignancy, anti-phospholipid syndrome, pregnancy and/or prescribed antiplatelet or anticoagulant medicines other than aspirin. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center. All patients and healthy subjects gave written informed consent for participation according to the declaration of Helsinki.

### Blood Collection

Blood was drawn from subjects in resting condition, and collected into 10 mL tubes containing 3.2% trisodium citrate. Portions of the whole-blood were directly used for flow cytometric and flow chamber assays. Separate blood samples were collected into 4 mL EDTA tubes (BD Vacutainer, Breda, the Netherlands) for determination of haemoglobin and blood cell counts with a Sysmex XN-9000 analyzer (Kobe, Japan).

### Whole-Blood Flow Cytometry

Agonist-induced platelet activation was assessed by an optimized and validated test using diluted whole-blood samples without centrifugation steps [22]. In brief, a 96-well plate was thawed, containing phycoerythrin (PE)-conjugated anti-P-selectin mAb (BD 555524, Pharmingen, Franklin Lakes, NJ) and FITC-conjugated anti-fibrinogen Ab (F011102, Dako, Denmark) together with varying concentrations of ADP (01897-1G, Sigma-Aldrich, Zwijndrecht, the Netherlands), collagen peptide (CRP-xI, Cambridge University, UK, a generous gift) [22] or TRAP-6 (H2936.0025, Bachem, Weil am Rhein, Germany); volume was 50  $\mu$ L in buffer A (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>). Activations were started by adding to each well 5  $\mu$ L of blood, while mixing. Final agonist concentrations were: 0.1–2.7  $\mu$ M ADP, 5–135 ng/mL collagen peptide or 0.4–3.2  $\mu$ M TRAP-6. A separate row did not contain platelet agonists and served as vehicle. After 20 minutes of activation, 500  $\mu$ L fixation solution (0.9% NaCl, 0.2% formaldehyde) was added to each well. Integrin activation and P-selectin expression in platelets were analyzed on an Accuri C6 flow cytometer (Becton and Dickinson, CA; 10,000 events), as described [24]. Data are represented as mean fluorescence intensities.

### Whole-Blood Thrombus Formation on Microspots

Whole-blood thrombus formation under flow was investigated with the Maastricht flow chamber (depth 50  $\mu$ m, width 3 mm, length 30 mm), employing a newly developed and validated multi-parameter assay in combination with three microspots [28]. Where indicated, control blood samples were pre-incubated with lysine acetylsalicylate for 10 minutes at 37 °C (100  $\mu$ M, Aspegic, Sanofi, Gouda, the Netherlands). Microspots on glass coverslips were prepared by applying 0.5  $\mu$ L collagen type I Horm (100  $\mu$ g/mL), von Willebrand factor (vWF, 50  $\mu$ g/mL) combined with CLEC-2 agonist rhodocytin (250  $\mu$ g/mL), and vWF plus fibrinogen (250  $\mu$ g/mL). Sources of microspots were as described before [32]. Citrate whole-blood perfusion was at arterial wall-shear rate of 1600 s<sup>-1</sup> for 3.5 minutes. Brightfield images were taken from all three microspots, while chambers were stained with Alexa Fluor (AF) 647-labeled annexin A5 (1:200, A23204, Life Technology, Eugene, OR) and FITC-labelled anti-P-selectin mAb (1:40, A07790, Beckman Coulter, Woerden, the Netherlands) in buffer B (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 0.1% glucose, 0.1% bovine serum albumin, 5 U/mL fragmin (low-molecular weight heparin) (RVG20607, Pfizer, Capelle a/d IJssel) and 1 U/mL unfractionated heparin (1760 U/mL, 125 K1336, Sigma), pH 7.45). The anticoagulants were added to all Ca<sup>2+</sup>-containing perfusion buffers to prevent thrombin and fibrin generation. Images of FITC and AF647 fluorescence were captured after a short rinse with buffer

**Table 1**

Baseline characteristics of control subjects and PAD patients. Data are represented as medians (ranges).

Subject characteristics	Controls	Patients	P
<b>General</b>			
Age, years	67 (60 – 71)	67 (65 – 72)	
Male gender, n	22/40	24/40	
Body mass index, kg/m <sup>2</sup>	26 (23.4 – 27.8)	26.1 (23.7 – 29.3)	
<b>Diagnosis PAD (Fontaine), n</b>			
I, IIa/IIb, III	0, 0, 0	1, 37, 2	
<b>Medical history, n</b>			
Diabetes mellitus	1	5	
Hypertension	13	29	
Aspirin intake	0	38	
Anticoagulant intake	0	0	
<b>Hemostatic variables</b>			
Platelet count, $\times 10^9/L$	255 (205 – 291)	249 (213 – 284)	n.s.
Mean platelet volume, fL	10.8 (10.2 – 11.4)	11.1 (10.4 – 11.9)	n.s.
Hematocrit, L/L	0.44 (0.42 – 0.47)	0.43 (0.39 – 0.45)	n.s.
Leukocyte count, $\times 10^9/L$	6.15 (5.60 – 7.45)	7.50 (6.03 – 9.13)	0.003
D-dimers, ng/mL	366 (259 – 520)	528 (363 – 835)	0.005

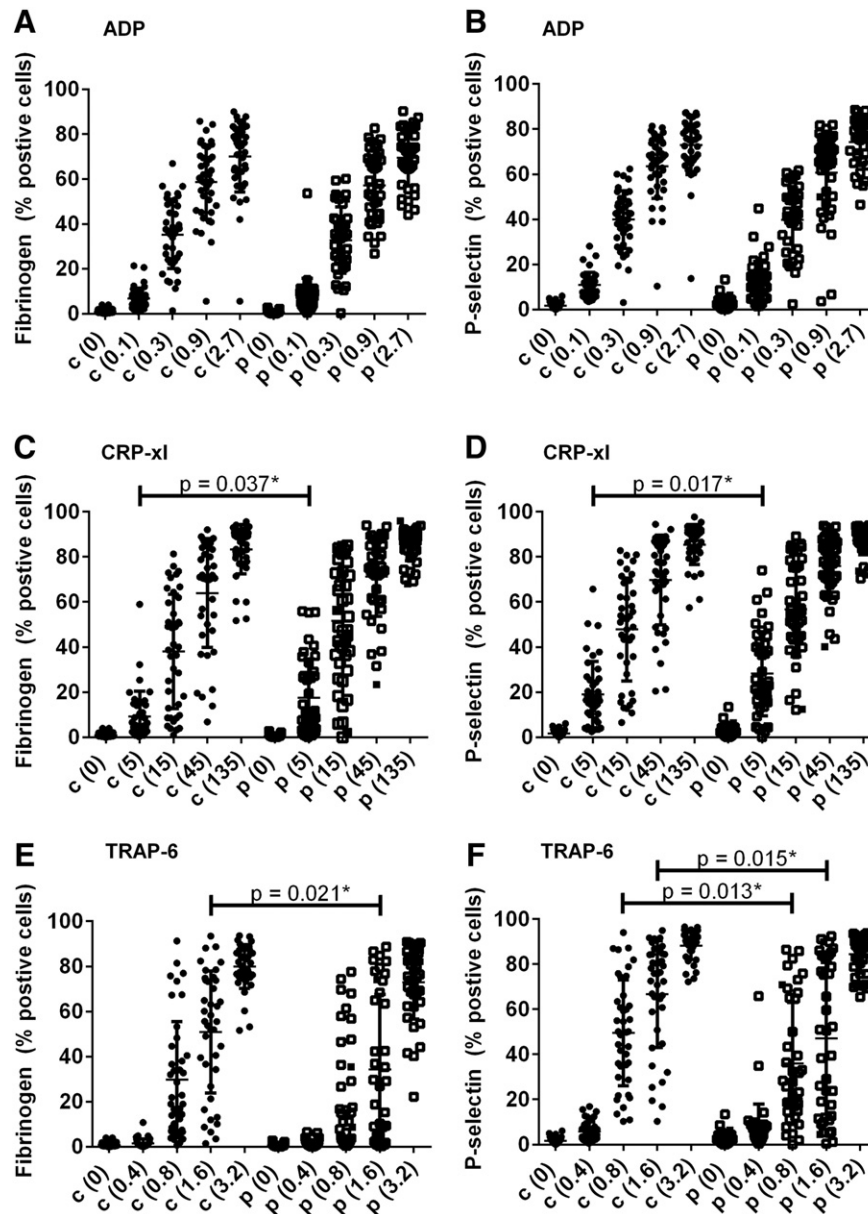
B. For microscopic imaging, an inverted EVOS fluorescence microscope (Life Technology) was used, basically as described elsewhere [28].

Whole-blood thrombus formation under flow was also assayed in real-time using a microfluidics polydimethyl siloxane (PDMS) flow chamber with a straight 1 mm wide x 50  $\mu$ m height channel, principally as described [33], but without stenosis. The disposable chamber was prepared with a Sylgard 184 kit, by mixing 43 g of silicone elastomer with 7 g curing agent (Dow Corning, Wiesbaden, Germany). The degassed mixture was poured into a round mold containing water to harden at room temperature. The flow channel inlet and outlet were made using a 3 mm biopsy punch and a blunt syringe needle, respectively. The flow chip was mounted onto a glass coverslip, coated with collagen type I Horm (100  $\mu$ g/mL) [34], fixed with a pressure plate, and pre-rinsed. Whole-blood perfusion, using a pulse-free syringe pump (pull mode), was performed as described [35]. Blood samples

(300  $\mu$ L) were pre-labeled with 0.5  $\mu$ g/mL DiOC<sub>6</sub> (6975, AnaSpec, San Jose, CA), and flowed at a wall-shear rate of 1600 s<sup>-1</sup> for 4 minutes. Microscopic images of deposition of fluorescently labeled platelets were taken as previously described [28], and analyzed for increases in fluorescence.

#### Microscopic Imaging and Analysis

Phase-contrast images were analyzed for surface-area-coverage and integrated feature size, using a standardized journal in Metamorph software [36]. Images were also assessed for morphological score from 0 (no platelet adhesion) to 5 (large platelet thrombi) [28]. Fluorescence images of antibody staining were analyzed for surface-area-coverage above background with the program Image J (open access).



**Fig. 1. Activation tendency of platelets from PAD patients and control subjects.** Diluted whole-blood from control subjects (c) or PAD patients (p) was stimulated in plate-wells with indicated doses of 0.1–2.7  $\mu$ M ADP (A, B), 5–135 ng/mL CRP-xI (C, D) or 0.4–3.2  $\mu$ M TRAP-6 (E, F). Binding of fibrinogen to the platelets (left panels) and P-selectin expression of platelets (right panels) were determined by flow cytometry, using a FITC-anti fibrinogen and a PE-anti-P-selectin mAb, respectively. Indicated are per subject the percentages of positively stained platelets. Patients using aspirin are represented by open squares. Bars indicate mean and S.D. values (n = 40), \*P < 0.05.



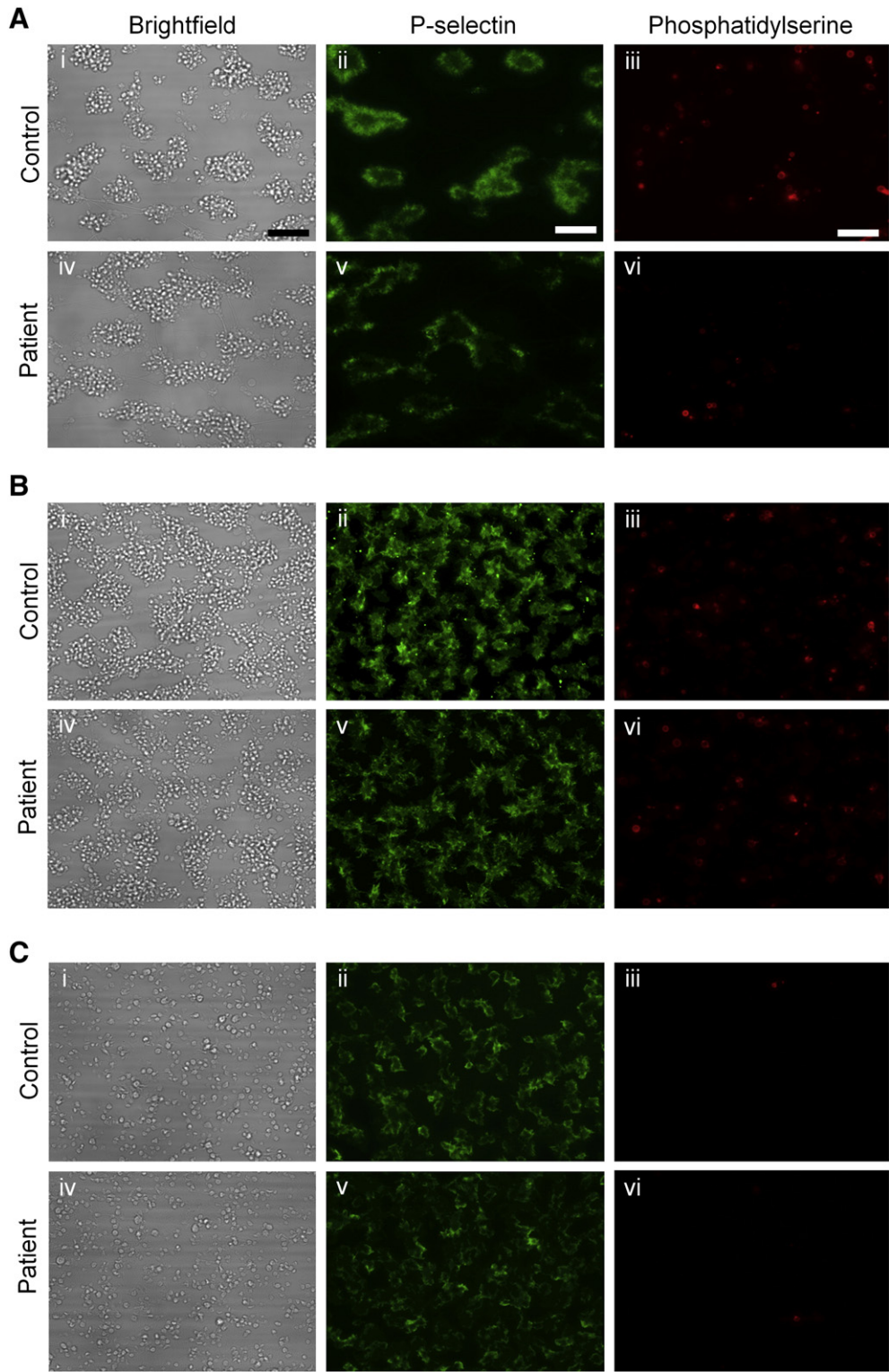
Statistics

Group comparisons were performed using the Mann-Whitney U-test for continuous variables. Assay parameters were compared using the Spearman correlation test. The statistical package for the Social Sciences version 22 was used (SPSS, Chicago, IL). *P*-values <0.05 were considered to be significant.

Results

Undiminished Platelet Activation in Whole-Blood from PAD Patients on Aspirin

Baseline characteristics of the 40 control subjects and 40 PAD patients were comparable for age, gender and body mass index (Table 1). With



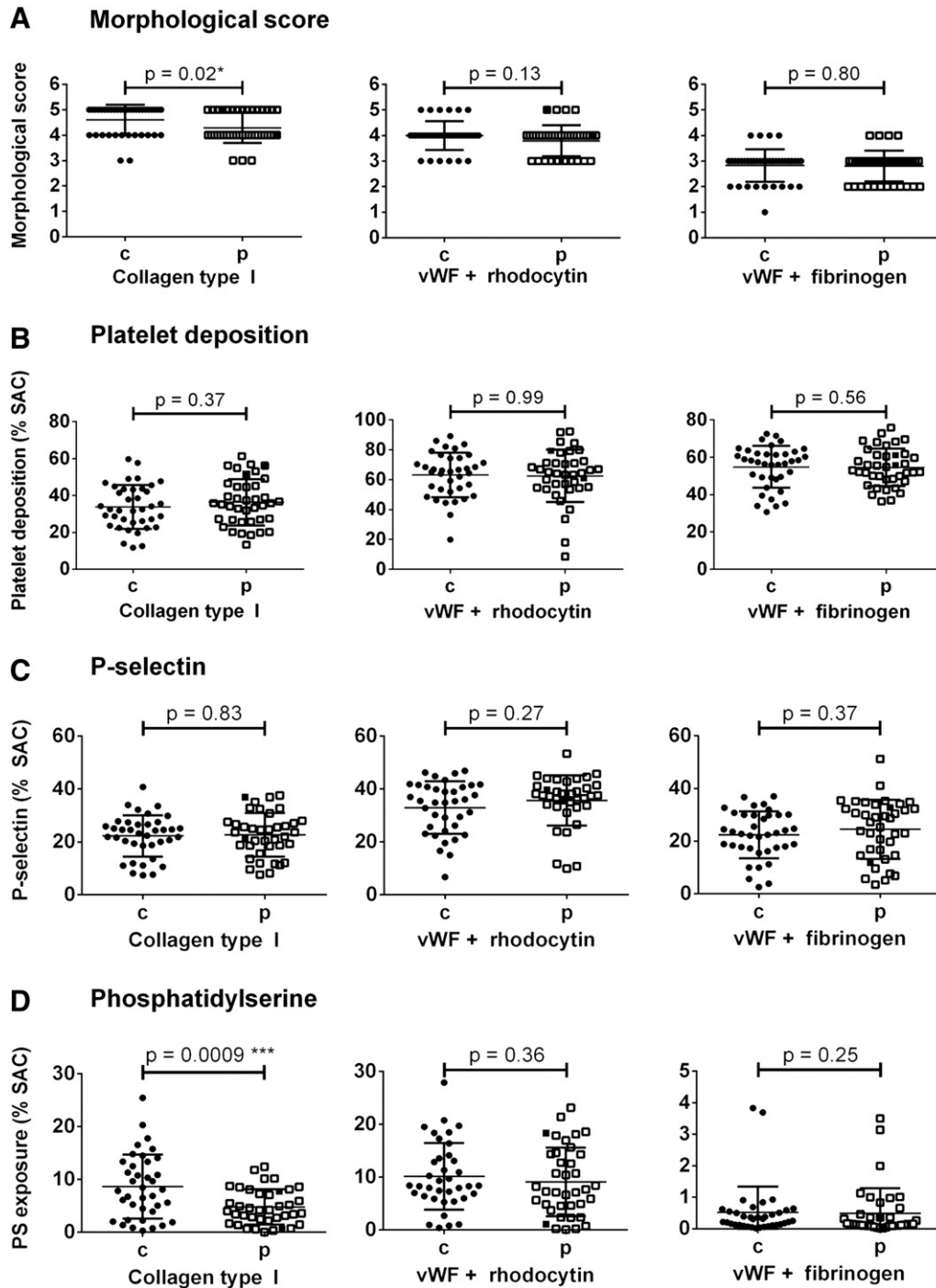
**Fig. 2.** Thrombus formation with blood from patients and controls at microspot surfaces. Whole blood from control subjects or patients was perfused for 3.5 minutes at wall shear rate of  $1600\text{ s}^{-1}$  over microspots containing collagen type I (A), vWF/rhodocytin (B), and vWF/fibrinogen (C). Shown are typical brightfield images (left) and fluorescence images of P-selectin expression (middle) or phosphatidylserine exposure (right) from a representative control subject and patient. Bars =  $20\text{ }\mu\text{m}$ .

respect to the medical history, the patients (vs. controls) more frequently suffered from diabetes mellitus (5 vs. 1) and hypertension (29 vs. 13), and in vast majority used prescribed aspirin (38 vs. 0). All patients, and none of the control subjects, were diagnosed as PAD with confirmed Fontaine classification IIa/IIb (37 out of 40). Most hemostatic variables of the PAD patients were in the normal range, but leukocyte counts and plasma levels of the coagulation product, D-dimers, were significantly increased in the patient group (Table 1).

Platelet activation tendency was measured in diluted whole blood samples, using a validated, 96-plate-well-based flow cytometric test,

in which both integrin  $\alpha_{IIb}\beta_3$  activation and P-selectin exposure are determined in response to dose ranges of the key agonists, ADP (stimulating P2Y<sub>12</sub>/P2Y<sub>1</sub> receptors), CRP-xI (stimulating the collagen receptor glycoprotein VI) and TRAP-6 (stimulating the thrombin receptor PAR1) [22]. For various agonists, intra- and inter-individual variation coefficients of the test were <10% and <15%, respectively, for the area under the curve [37].

In response to the weak agonist ADP, platelets from patients and control subjects showed a dose-dependent increase in integrin activation (fibrinogen binding) and limited P-selectin expression (Fig. 1A,



**Fig. 3. Parameters of thrombus formation with blood from patients and controls.** Whole blood from control subjects (c) and PAD patients (p) was perfused over coated microspots, as described for Fig. 2. Shown are analyzed data per subject of brightfield images of morphological score (A) and platelet deposition (B); and of staining for P-selectin (C) and exposed phosphatidylserine (D). Patients using aspirin are represented by open squares. Bars indicate mean and S.D. values ( $n = 40$ ),  $^*P < 0.05$ .

B). Dose-response curves varied per subject but, overall, did not markedly differ between the patient and control groups. Platelet stimulation with CRP-xI (Fig. 1C,D) or TRAP-6 (Fig. 1E,F) resulted in dose-dependent increases in integrin activation and P-selectin expression that, with these stronger agonists, reached maximal levels at high doses. At lower CRP-xI concentrations, both integrin activation and P-selectin expression were significantly higher in the patient group than in the control group ( $P \leq 0.037$ ), but ranges were overlapping. In contrast, in response to higher doses of TRAP-6, integrin activation ( $P = 0.021$ ) and P-selectin expression ( $P \leq 0.015$ ) were significantly lower in the patient group compared to the controls. Two of the patients did not use aspirin. In blood samples from these patients, agonist-induced integrin activation and P-selectin expression were mostly but not always in the higher ranges (see Fig. 1). Upon stimulation with ADP, no significant effect of aspirin treatments was observed (not shown). This is in agreement with the known sensitivity for aspirin of glycoprotein VI-induced platelet responses [38]. Taken together, these data indicate that, in spite of the use of aspirin, the platelets from PAD patients were in general similarly high in response as the platelets from control subjects, with only reduced activity at high TRAP-6 doses.

#### Essentially Unchanged Thrombus Formation in Patient Whole Blood

Whole-blood thrombus formation was tested on three microspots, representing adhesive ligands for major platelet receptors, *i.e.* collagen type I (activating via glycoprotein VI and integrin  $\alpha_2\beta_1$ ), vWF/rhodocytin (acting via GPIb and CLEC-2 receptors), and vWF/fibrinogen (acting via GPIb and integrin  $\alpha_{IIb}\beta_3$ ). The presence of vWF (also binding to collagen) allowed thrombus formation to be assessed at high, arterial shear rates [28]. On collagen, large platelet aggregates were formed, staining positively for P-selectin, and individual platelets in the thrombus were present, staining positively with annexin A5 for phosphatidylserine exposure (Fig. 2A), as established before [39]. On vWF/rhodocytin smaller aggregates formed, while staining for P-selectin and phosphatidylserine exposure was still high (Fig. 2B). On vWF/fibrinogen mostly single platelets adhered with P-selectin but no phosphatidylserine exposure (Fig. 2C). Principal assay parameters per surface were obtained following quantitative analysis of the recorded images, according to standard protocols [36]. These were: morphological score, platelet deposition (surface-area-coverage of brightfield images), integrated feature size (weighted cumulative size of platelet aggregates), and extent of P-selectin and phosphatidylserine exposure (Fig. 3A–D). In agreement with earlier assessment of high inter-individual variation of most of these parameters [28], values obtained per subject (controls and patients) showed large differences. Markedly, however, only few differences in these parameters were detected between the patient

and control groups. With the patient blood samples, thrombi on collagen were lower in morphological score, integrated feature size and phosphatidylserine exposure ( $P \leq 0.02$ ). None of the parameters differed for thrombi formed on vWF/rhodocytin or vWF/fibrinogen.

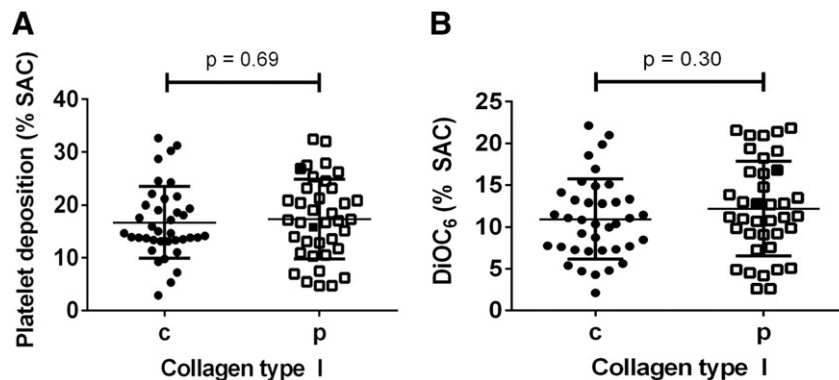
Furthermore, real-time imaging of platelet adhesion to collagen using DiOC<sub>6</sub>-labeled blood samples did not show differences between the control group and patient group (Fig. 4A,B). Similarly, as reported before [28], markers of thrombus formation – per surface and per patient/control sample – were correlated (data not shown). Together, this indicated that for the patient group platelet adhesion, activation and aggregation under flow was mostly unaltered compared to the healthy, control group.

*In vitro* studies using blood samples from healthy controls were carried out to specifically determine the effect of aspirin on the various assay parameters. As indicated in Fig. 5, on collagen and to a lesser extent on vWF/rhodocytin (morphological score and phosphatidylserine exposure), but not on vWF/fibrinogen, aspirin treatment reduced aspects of thrombus formation. Especially on collagen, thrombus parameters were reduced by more than half.

Comparing the various parameters of collagen-dependent thrombus formation per subject (patients and controls), gave high correlations ( $P < 0.001$ ,  $r^2 = 0.25$ – $0.56$ , Spearman). This compares well with a previous systematic analysis of the thrombus-forming process with blood from healthy subjects [28], and confirms that – in the absence of platelet aberrations – the various platelet activation responses are highly linked. Furthermore, compared per subject, markers of ADP- and collagen-induced integrin activation (flow cytometry) strongly correlated with parameters of collagen-dependent platelet aggregation under flow (Table 2), pointing to a subject-dependent activation factor commonly regulating both types of platelet responses.

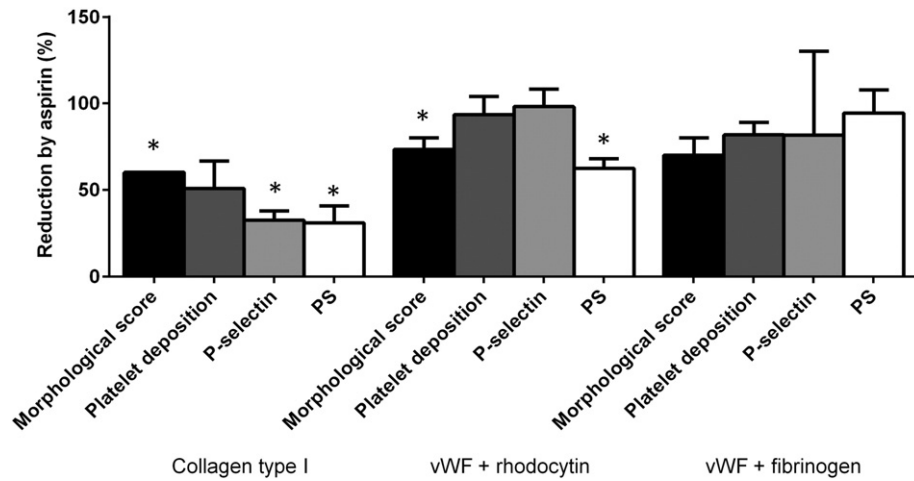
#### Discussion

A recent meta-analysis of randomized trials indicates that in patients with PAD, treatment with aspirin alone led to a statistically insignificant decrease in cardiovascular events, but a significant reduction in non-fatal stroke [40]. Several small-size studies have pointed to high activity of platelets in PAD patients taking aspirin [20,21], which can be reversed by the antiplatelet agent iloprost, a prostacyclin analogue [19]. Here, we used high-throughput whole-blood assays, *i.e.* multi-agonist testing of platelet activation by flow cytometry and multi-parameter testing of thrombus formation on microarray spots, to determine the platelet activation tendency in 40 patients with PAD receiving aspirin compared to healthy controls not on antiplatelet medication. This systematic analysis points to a general high on-treatment function of the patient platelets in thrombus formation, and thus supports the hypothesis that the



**Fig. 4.** Flow-dependent adhesion of platelets with blood from patients and controls. Whole blood pre-treated with DiOC<sub>6</sub> from control subjects (c) and PAD patients (p) was perfused through a collagen-coated PDMS chamber for 3.5 minutes at wall shear rate of  $1600 \text{ s}^{-1}$ . Analyzed data per subject of brightfield images of platelet deposition (A) and integrative adhesion of DiOC<sub>6</sub>-labeled platelets (B). Patients using aspirin are represented by open squares. Bars indicated mean and S.D. values ( $n = 40$ ).





**Fig. 5.** Effect of aspirin on whole-blood thrombus formation at microspot surfaces. Whole blood from control subjects was perfused over coated microspots, as in Fig. 2. Blood samples were incubated with vehicle or lysine acetylsalicylate (100  $\mu$ M) for 10 minutes at 37 °C prior to perfusion. Images were analyzed for morphological score, platelet deposition, staining for P-selectin and phosphatidylserine (PS), as described for Fig. 3. Data are expressed as percentages relative to vehicle control. Means  $\pm$  S.E.M. (n = 4), \*P < 0.05.

prothrombotic propensity known for PAD patients is not accompanied by an increased platelet activation profile after intake of aspirin.

In collagen-induced thrombus formation, platelet stimulation via glycoprotein VI and ADP increases the affinity state of integrins  $\alpha_2\beta_1$  and  $\alpha_{IIb}\beta_3$  [41,42], and leads to stable platelet adhesion and aggregate

formation [39]. Given that ADP is a primary agonist in establishing stable platelet-platelet interactions under flow [43], the similarity in thrombus formation between the patient and control groups matches the comparable ADP-induced integrin activation (fibrinogen binding) in both groups determined by flow cytometry. This notion is confirmed by the observation that markers of ADP- and collagen-receptor dependent  $\alpha_{IIb}\beta_3$  activation (flow cytometry) strongly correlated with markers of the thrombus-forming process on collagen, which is indicative of a common, subject-dependent regulation of both processes.

Although platelet activation and thrombus parameters in the patient group were generally comparable with the control group, there were some typical differences versus the healthy control group. The indication for increased platelet activity in PAD patients came from flow cytometric analysis of platelet activation with low but not high CRP-xI concentrations (stimulating glycoprotein VI). In contrast, decreased activity in the PAD group was observed for TRAP-6-induced platelet activation (stimulating thrombin receptors), which is an aspirin-sensitive response. Furthermore, we found moderate reductions in some (morphological score, phosphatidylserine exposure), but not all (P-selectin and platelet deposition) parameters of thrombus formation on collagen in the patient group. Although these can be explained by the use of aspirin, the effect size appears to be smaller than observed by direct and complete inhibition of control platelets with aspirin *in vitro*. In agreement with the present results, others have also shown that aspirin markedly affects thrombus formation on collagen under flow [44,45].

The seeming contrasting differences between increased stimulation of patients' platelets with low doses of CPR-xI and reduced parameters of collagen-dependent thrombus formation, can be explained by the fact that, in thrombus formation under flow, not only glycoprotein VI, but also other platelet receptors like integrin  $\alpha_2\beta_1$  and GPIb-V-IX are determinative for this process [46]. This suggests a partly diminished role of these other receptors in the patient group, concerning morphological score and phosphatidylserine exposure on collagen. The effect on phosphatidylserine exposure yet is relevant, because this platelet response is a major trigger of coagulation factor binding and thrombin formation on the platelet surface [47].

Strengths of this study are the relatively large size of the PAD patients (class IIa/IIb) and control groups; the use of well standardized tests using whole blood samples with no platelet activation due to centrifugation steps; and the ability to evaluate multiple platelet activation processes and markers in the same tests. Given the overall consistency of the various assay results, this study may help in improving the management and treatment of PAD, e.g. by considering more effective anti-thrombotic pharmacoprotection.

**Table 2**

Correlations of agonist-induced fibrinogen binding and P-selectin expression with parameters of thrombus formation on collagen microspots. Compared are analyses of platelet deposition (brightfield % surface area coverage, SAC), aggregate size (integrated feature size, IFS), and DiOC<sub>6</sub> staining. Spearman correlation analysis of data from all subjects (n = 80); given are two-sided P values per flow-cytometric data set. Colour scale from green to white: low to high P values.

Thrombus parameter	Fibrinogen binding			P-selectin expression		
	SAC	IFS	DiOC <sub>6</sub>	SAC	IFS	DiOC <sub>6</sub>
ADP (0.1)	0.056	0.014	0.323	0.417	0.929	0.394
ADP (0.3)	0.036	0.014	0.125	0.226	0.424	0.599
ADP (0.9)	0.016	0.007	0.058	0.096	0.232	0.239
ADP (2.7)	0.041	0.064	0.164	0.321	0.665	0.558
ADP (AUC)	0.033	0.008	0.202	0.243	0.361	0.664
CRP-xI (5)	0.007	0.011	0.058	0.346	0.336	0.466
CRP (15)	0.013	0.030	0.012	0.104	0.198	0.882
CRP-xI (45)	0.083	0.050	0.066	0.393	0.219	0.310
CRP-xI (135)	0.072	0.083	0.107	0.579	0.805	0.627
CRP-xI (AUC)	0.048	0.033	0.040	0.188	0.161	0.176
TRAP-6 (0.4)	0.023	0.001	0.077	0.805	0.781	0.775
TRAP-6 (0.8)	0.280	0.032	0.011	0.649	0.198	0.083
TRAP-6 (1.6)	0.152	0.090	0.073	0.836	0.844	0.436
TRAP-6 (3.2)	0.096	0.340	0.015	0.608	0.904	0.149
TRAP-6 (AUC)	0.162	0.019	0.008	0.565	0.484	0.104
All agonists (ΣAUC)	0.070	0.048	0.064	0.017	0.012	0.012



## Conflict of Interest

The authors have declared that no competing interests exist.

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